sequencing methods are available, they suffer from significant limitations, including the need for sophisticated instrumentation, microfluidics, or nanofabrication. Here, we introduce a simpler approach based on fluorescently labelled DNA polymerases and dark quencher labelled nucleotides (dNPP-Q, pentaphosphates). During the time between the binding and incorporation of a nucleotide, the fluorescence intensity of the polymerase-attached fluorophores is differentially reduced due to Foerster resonance energy transfer to a nucleotide-specific dark-quencher attached to the terminal phosphate group. Use of dark-quenchers enables real-time sequencing with long read lengths at micromolar nucleotide concentrations, and is compatible with standard total-internal-reflection fluorescence microscopy.
We first characterized dark quenchers for single-molecule detection by studying a set of double-stranded DNA constructs labelled with two fluorophores (''green'' and ''red'') and one dark-quencher. We then designed a biochemical system that allowed us to observe directly individual events of dNPP-Q binding to a binary complex of a DNA polymerase (labelled with a green fluorophore) bound to a primer-template substrate labelled with a red fluorophore. Binding events of dNPP-Q to the complex results in real-time observations of clear, transient reductions of green and red fluorescence by 80% and 30%, respectively; the quenching efficiency matches to the expectations based on the photophysical properties of the interacting chromophores and their separation. We are currently extending the concept to the remaining bases. Our approach should facilitate the study of DNA- and RNA-polymerase mechanisms as well as the development of faster and cheaper methods for single-molecule DNA sequencing.

3177-Plat
Photo-Control of Protein Activity in a Single Cell of a Live Organisim
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We take a multidisciplinary approach to realize a noninvasive optical control of the protein activity in live zebrafish embryo with single cell resolution. Our method relies on three components 1) the dependence of biological activity of the protein fused to estrogen receptor on the binding state of the estrogen to its chaperone, 2) the chemical inertness and the permeability of the cell membrane to the non-endogeneous caged inducer and 3) the selective optical uncaging of the inducer in the zebrafish embryo, globally or in a single cell using one or two photon microscopy. We believe that our method is very general and could be used to control the activity of large array of proteins. As a pilot study we used our method to activate 1) the nuclear translocation of two different fluorescent proteins in embryo and cell cultures, and 2) the Cre recombinase activity in an appropriate transgenic animal to genetically label a single cell of the embryo and cell lines. The ability of our method to change the genetic map of the selective cells in the embryos could be used more generally to investigate important physiological processes (for example in embryogenesis, organ regeneration and carcinogenesis) with high spatio-temporal resolution (single cell and faster than minute scales).

3178-Plat
Genetically Encoded Singlet Oxygen Generator (SOG) Requiring No Exogenous Cofactors
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Controlled local photogeneration of singlet oxygen (1O2, the metastable excited state of O2) is useful for generating electron-microscopic contrast, rapidly inactivating proteins of interest, reporting protein proximities over tens of nanometers, and ablating cells by photodynamic damage. The best previous genetically targetable SOG was the biarsenical dye ReAsH bound to tetracysteine motifs, but this system has modest quantum efficiency (0.024), requires antidotes to prevent toxicity, is limited by background staining, and is difficult to extend to intact multicellular organisms. We now report that Arabidopsis phototropin, a blue light photoreceptor containing flavin mononucleotide (FMN) as its chromophore, can be engineered into a small (106-residue) SOG (''miniSOG''), which absorbs maximally at 448 and 473 nm with extinction coefficients of 16,700 and 13,600 M-1cm-1 respectively. Quantum yields for fluorescence and 1O2 generation are 0.30 and 0.47. MiniSOG binds endogenous FMN very tightly (dissociation constant ~ 10-10 M), so bacteria and mammalian cells upregulate their total FMN to keep miniSOG saturated, without any obvious toxicity in the absence of illumination. Although the green fluorescence of miniSOG is weak and bleachable, it shows that fusions of miniSOG to a variety of proteins in mammalian cells appear to localize correctly, even inside organelles when appropriate. After fixation, illumination of miniSOG to generate 1O2 efficiently polymerizes diaminobenzidine into an osmiophilic deposit, enabling correlative electron microscopy. In an initial biological application, electron microscopy shows that a cell-adhesion molecule, SynCAM1, fused to miniSOG, predominantly localizes to the presynaptic side of cortical neuron synapses. This compact SOG relying only on ubiquitous endogenous FMN will greatly expand the utility of imaging and ablation techniques based on 1O2.

Platform AV: Microtubular Motors

3179-Plat
The Neck Linker Docking is Not Required for Kinesin-1 to Take a Step Forward
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Kinesin-1 moves processively along microtubules by alternatively moving two motor domains, but the mechanism of the preferential forward stepping is still controversial. The ''neck linker-docking model'' proposes that the neck linker of the microtubule-bound head propel the tethered head forward. We proposed an alternative ''biased-capturing model'' that the tethered head freely diffuses and is captured preferentially at the forward-binding site. The latter model predicts that the neck linker of the tethered head, not of the microtubule-bound head, is essential for stepping, but it is difficult to distinguish these effects using ''symmetric kinesin dimer''. To distinguish these models, we engineered ''asymmetric two-headed monomer'' kinesin. We joined two monomer heads tandemly on a single polypeptide, in which the neck linker of first head (Nhead) is connected to second head (Chead) so that it can propel Chead forward, whereas the neck linker of Chead is free and is not connected to Nhead. The neck linker-docking model would predict this mutant could not take the second step. Surprisingly, the two-headed monomer showed robust and unidirectional movement along microtubules in single molecule fluorescent assays. The distance travelled was even longer than wild-type dimer but the velocity was reduced by a factor of 4. Single molecule FRET observation showed that the mutant spent most of the time in a two-head-bound state where the Nhead is leading. These results indicate that the rate-limiting step of the two-headed monomer's processive movement is the forward stepping of Chead driven by the neck linker-docking of Nhead and that the neck linker-docking independent forward stepping of Nhead is rapid and more efficient. These results rule out the idea that neck linker docking is essential to take a forward step and favour the biased-capturing mechanism.

3180-Plat
Possible Intermediate States in the Microtubule Minus-End Directed Movement of the Ncd Stalk
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The walking step of the microtubule minus-end directed kinesin motor Ncd involves about 75-degree rotation of its coiled-coil stalk. Two crystal structures, 1CZ7 and 1N6M (protein data bank IDs) are related respectively to conformations before (plus-end pointing stalk) and after (minus-end pointing stalk) this movement. Using targeted molecular dynamics we traced the stalk motion between these two structures. We observed that the motor head first rearranges from the 1CZ7 to 1N6M conformation, followed by the travel of the stalk. The 75-degree rotation of the stalk is accompanied by a nearly 20-degree torsion in its coiled coil. Further, the stalk travel can be divided into sub-steps between intermediates at about 17, 38 and 56 degrees from the 1CZ7 conformation that are characterized by the breaking and forming of new salt bridges by Arg335 or Lys336 in the stalk with the charged groups along the alpha-1 domain of the motor head, some of which are not present in the crystal structures. These results suggest that when Ncd is making a step towards the microtubule minus-end, the motor head first changes conformation in a nucleotide-dependent manner that promotes the detachment of the plus-end pointing stalk. This is followed by the stalk rotation that may be broken down into sub-steps between the intermediate states. In case of a diffusive motion, this may be more effective than diffusion over the entire 75-degree range.